Development/Plasticity/Repair

Induction of Proneurotrophins and Activation of p75^{NTR}-Mediated Apoptosis via Neurotrophin Receptor-Interacting Factor in Hippocampal Neurons after Seizures

Marta Volosin,[1] Christy Trotter,[1] Andrea Cragnolini,[1] Rajappa S. Kenchappa,[2] Matthew Light,[3] Barbara L. Hempstead,[3] Bruce D. Carter,[2] and Wilma J. Friedman[1]

[1]Department of Biological Sciences, Rutgers University, Newark, New Jersey 07102, [2]Department of Biochemistry, Vanderbilt University, Nashville, Tennessee 37232, and [3]Weill Medical College of Cornell University, New York, New York 10021

Seizure-induced damage elicits a loss of hippocampal neurons mediated to a great extent by the p75 neurotrophin receptor (NTR). Proneurotrophins, which are potent apoptosis-inducing ligands for p75^{NTR}, were increased in the hippocampus, particularly in astrocytes, by pilocarpine-induced seizures; and infusion of anti-pro-NGF dramatically attenuated neuronal loss after seizures. The p75^{NTR} is expressed in many different cell types in the nervous system, and can mediate a variety of different cellular functions by recruiting specific intracellular binding proteins to activate distinct signaling pathways. In this study, we demonstrate that neurotrophin receptor-interacting factor (NRIF) mediates apoptotic signaling via p75^{NTR} in hippocampal neurons *in vitro* and *in vivo*. After seizure-induced injury, *NRIF*$^{-/-}$ mice showed an increase in p75^{NTR} expression in the hippocampus; however, these neurons failed to undergo apoptosis in contrast to wild-type mice. Treatment of cultured hippocampal neurons with proneurotrophins induced association of NRIF with p75^{NTR} and subsequent translocation of NRIF to the nucleus, which was dependent on cleavage of the receptor. Neurons lacking NRIF were resistant to p75^{NTR}-mediated apoptosis *in vitro* and *in vivo*. In addition, we demonstrate some mechanistic differences in p75^{NTR} signaling in hippocampal neurons compared with other cell types. Overall, these studies demonstrate the requirement for NRIF to signal p75^{NTR}-mediated apoptosis of hippocampal neurons and that blocking pro-NGF can inhibit neuronal loss after seizures.

Key words: **neurotrophins; p75 neurotrophin receptor; apoptosis; signal transduction; NRIF; hippocampus**

Introduction

The p75 neurotrophin receptor (p75^{NTR}) can mediate many diverse cellular functions such as survival, apoptosis, and axonal growth. These different functions depend on cellular context, the stimulating ligand, association of coreceptors, and recruitment of intracellular binding proteins to activate specific signaling pathways. Proneurotrophins binding to p75^{NTR} can induce apoptosis after injury (Harrington et al., 2004; Volosin et al., 2006) or disease (Peng et al., 2004; Pedraza et al., 2005). The apoptotic activity of p75^{NTR} induced by proneurotrophins requires interaction with sortilin as a coreceptor (Nykjaer et al., 2004; Teng et al., 2005; Volosin et al., 2006), and signals via the intrinsic caspase pathway, requiring phosphorylation of c-Jun N-terminal kinase (JNK) (Friedman, 2000) and activation of caspases-9, -6, and -3 (Troy et al., 2002), in contrast to other death receptors that signal via the extrinsic, caspase-8-dependent pathway (Green, 1998). The p75^{NTR} can also interact with Trk receptors to facilitate survival and differentiation (Hempstead et al., 1991), although the exact nature of this interaction is unclear (Wehrman et al., 2007). In addition, p75^{NTR} is part of the Nogo receptor signaling complex activated by myelin proteins to inhibit axonal growth (Wang et al., 2002; Wong et al., 2002). Despite accumulating evidence for the important roles played by p75^{NTR}, little is known about the mechanisms by which it activates specific signaling pathways. Although many proteins have been identified that can interact with the p75^{NTR} intracellular domain (ICD) (Gentry et al., 2004a), their roles in mediating specific functions in different cell types have not been fully elucidated, especially *in vivo*. Several p75^{NTR} binding proteins have been implicated as mediators of apoptotic signaling, such as neurotrophin receptor-interacting factor (NRIF) (Casademunt et al., 1999; Linggi et al., 2005), p75^{NTR}-associated death executor (NADE) (Mukai et al., 2000), neurotrophin receptor-interacting MAGE homolog (NRAGE) (Salehi et al., 2002), and necdin (Tcherpakov et al., 2002; Kuwako et al., 2004); however, determining the role of these proteins in mediating p75^{NTR}-dependent apoptosis in appropriate physiological conditions remains a major challenge. NRIF has been implicated in the p75^{NTR}-mediated apoptotic pathway based on phenotypic similarities between *nrif*$^{-/-}$ and *p75*$^{-/-}$ mice (Casademunt et al., 1999). Moreover, NRIF is required for p75^{NTR}-mediated apoptosis in sympathetic neurons (Kenchappa et al., 2006). This mechanism requires p75^{NTR} to be cleaved in the

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Correspondence should be addressed to Dr. Wilma J. Friedman, Department of Biological Sciences, Rutgers University, 225 University Avenue, Newark, NJ 07102. E-mail: wilmaf@andromeda.rutgers.edu.

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membrane by α- and γ-secretases (Jung et al., 2003; Zampieri et al., 2005), yielding an intracellular domain that contributes to signaling cell death (Kenchappa et al., 2006).

We have previously demonstrated that $p75^{NTR}$ mediates apoptosis of hippocampal and basal forebrain neurons in response to ligand treatment in culture, and after seizure-induced injury *in vivo* (Friedman, 2000; Troy et al., 2002; Volosin et al., 2006). In this study, we demonstrate that proneurotrophins, the preferred ligands for inducing $p75^{NTR}$-mediated apoptosis, are increased in the hippocampus and are required for neuronal loss after pilocarpine-induced seizures. Furthermore, we show that NRIF mediates $p75^{NTR}$-activated apoptosis of hippocampal neurons *in vitro* and after seizures *in vivo*. However, the time course and signaling mechanisms in hippocampal neurons differ from other cell types, such as sympathetic neurons, demonstrating cell specificity in $p75^{NTR}$ signaling.

Materials and Methods

Materials. NGF was generously provided by Genentech, and BDNF was a gift from C. F. Ibáñez (Karolinska Institute, Stockholm, Sweden). Anti-p75 (IgG 192) and OX-42 were purchased from Millipore Bioscience Research Reagents, anti-p75 9651 was generously provided by M. V. Chao (Skirball Institute, New York University, New York, NY), and anti-sortilin (anti-neurotensin receptor 3) antibody was from Alpha Diagnostics. Cleaved caspase-3 antibody was from Cell Signaling Technology, and Nucview caspase-3 substrate was from Biotium. Antibodies to the p75 ICD, affinity-purified and immunodepleted anti-NRIF were prepared as previously described (Kenchappa et al., 2006). Ubiquitin (sc-8017), TRAF6 (sc-8409), TRAF2 (sc-7346), NGF (H-20), and BDNF (N-20) antibodies were purchased from Santa Cruz Biotechnology. NRAGE antibody was from Millipore, and anti-GFAP was from Roche Biosciences. Eagle's MEM, Ham's F12, and penicillin–streptomycin were purchased from Invitrogen. Polylysine, glucose, insulin, putrescine, progesterone, transferrin, and selenium were obtained from Sigma-Aldrich. Secondary antibodies used for immunostaining were Alexa 488 and Alexa 555 anti-rabbit and anti-mouse antibodies purchased from Invitrogen. TAPI-1, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), and recombinant MMP-7 were purchased from Calbiochem.

Generation of viral vectors and recombinant murine pro-NGF. Generation of the furin-resistant pro-NGF was performed as described previously (Lee et al. 2001). Briefly, the cDNA encoding mouse NGF was amplified by reverse transcriptase-PCR and bidirectionally sequenced. Using PCR-based mutagenesis, six histidine (His) residues were added at the C terminus, and point mutation of residues KR (amino acids 120 and 121) and residues RR (amino acids 239 and 240) to AA was performed.

Recombinant baculoviral expression vectors were generated encoding the furin-resistant His-tagged pro-NGF using the Bac-to-Bac baculovirus expression system (Invitrogen). Baculoviral stocks were amplified and propagated using *Spodoptera frugiperda* (Sf9) cells cultured in Sf-900 II serum-free media for 72–96 h. All baculovirus expression system-related reagents and cells were from Invitrogen.

S. frugiperda (Sf9) cells were infected with a multiplicity of infection of 2 and cultured in Sf-900 II serum-free medium for 66 h. The medium was then harvested, and proteinase inhibitors were added (1 mM PMSF, 10 μg/ml leupeptin, and 1 μg/ml aprotonin). Media were subjected to tangential flow dialysis (Minimate TFF Capsule; Pall Corporation) against PBS, followed by purification in a HisTrap HP 1 ml column (GE Healthcare) and eluted with 20 mM Na-phosphate, 0.5 M NaCl, 1 M imidazole, pH 6.0. Recombinant proteins were dialyzed against PBS and stored at −80°C until use.

Pro-BDNF was purified from the media of 293 cells expressing the pro-BDNF plasmid as described previously (Lee et al., 2001; Teng et al., 2005).

Neuronal cultures. Pregnant rats or mice were killed by exposure to CO_2 and soaked in 80% ethanol for 10 min. Rat fetuses were removed at embryonic day 18 (E18) under sterile conditions and kept in PBS on ice. For experiments with mice, fetuses were removed from E16 Sv129 wild-type or $NRIF^{-/-}$ mice. The hippocampus was dissected, dissociated by trituration in serum-free medium, plated on polylysine (0.1 mg/ml)-coated tissue culture wells or plastic slide wells (Nalge Nunc International), and maintained in a serum-free environment (Friedman et al., 1993; Farinelli et al., 1998). Medium consists of a 1:1 mixture of Eagle's MEM and Ham's F12 supplemented with glucose (6 mg/ml), putrescine (60 μM), progesterone (20 nM), transferrin (100 μg/ml), selenium (30 nM), penicillin (0.5 U/ml), and streptomycin (0.5 μg/ml). In all experiments, neurons were cultured for 4–5 d before treatment. Cultures maintained under these conditions contained <2% glial cells, confirmed by staining for glial markers. The absence of glia is critical because astrocytes in culture produce NGF.

Neuronal survival assay. Survival of cultured hippocampal neurons was assayed by lysing the cells and counting intact nuclei using a hemacytometer, as we have done previously (Farinelli et al., 1998; Maroney et al., 1999; Friedman, 2000). Nuclei of dead cells either disintegrate, or if in the process of dying, appear pyknotic and irregularly shaped. In contrast, nuclei of healthy cells are phase bright and have clearly defined membranes. Cell counts were performed in triplicate wells. Statistical significance was determined by ANOVA with Tukey's *post hoc* analysis.

Immunoprecipitation and Western blot analysis. Cells were lysed in a buffer consisting of Tris-buffered saline with 0.1% Triton, 60 mM octylglucoside, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM sodium vanadate. Total protein was quantified by the Bradford assay (Bio-Rad). For p75 or NRIF immunoprecipitation, 150–200 μg of total protein from hippocampal neuron lysates was incubated with monoclonal antibody 192 IgG (Millipore Bioscience Research Reagents) or affinity-purified anti-NRIF overnight on a rocking platform at 4°C. Protein G- or protein A-Sepharose (30 mg/ml; Pfizer) was then added to the lysates and kept for an additional 2 h at 4°C. Immunoprecipitates were washed three times with lysis buffer and one time with water, and subjected to Western blot analysis and probed with antibodies to NRIF (Gentry et al., 2004b) or p75 (Huber and Chao, 1995). Blots were stripped and reprobed with the 9651 anti-p75 antiserum or anti-NRIF. Additionally, lysates were immunoprecipitated with anti-NRIF, followed by Western blotting with anti-NRAGE, anti-TRAF2, or anti-TRAF6.

For NRIF ubiquitination studies, cultured hippocampal neurons were treated with vehicle or pro-NGF for 30 min. Cells were lysed with SDS lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1% SDS, and proteinase inhibitors), incubated on ice for 15 min, and then sonicated and centrifuged. Supernatants were immunoprecipitated with an affinity-purified NRIF antibody and subjected to Western blot analysis for ubiquitin.

Electrophoretic mobility shift analysis. Nuclear extracts were prepared from control, NGF, or tumor necrosis factor α (TNFα)-treated hippocampal neurons by a modification (Friedman et al., 1996) of the method of Dignam et al. (1983). A total of 5 μg of nuclear protein was incubated with ^{32}P-labeled oligonucleotides with the consensus sequence for nuclear factor-κB (NF-κB). Lysates of MT4 cells were used as positive control. Competition with excess unlabeled oligonucleotides confirmed the specificity of the shifted bands. DNA–protein complexes were resolved by electrophoresis through a 4.5% polyacrylamide gel under nonreducing conditions. Each gel is representative of at least three independent experiments.

Pilocarpine-induced seizures. Male Sprague Dawley rats (250–275 g) were pretreated for 0.5 h with methyl-scopolamine (1 mg/kg, s.c.; Sigma-Aldrich), and then treated with pilocarpine hydrochloride (350 mg/kg, i.p.; Sigma-Aldrich). After 1 h of status epilepticus, rats were treated with diazepam (10 mg/kg; Abbott Laboratories) and phenytoin (50 mg/kg; Sigma-Aldrich) to stop seizure activity. Additional diazepam was administered as necessary to prevent additional seizures. Adult mice (24–30 g) were also pretreated for 0.5 h with methyl-scopolamine and, in addition, were pretreated with phenytoin (50 mg/kg; Sigma-Aldrich) to prevent mortality associated with tonic seizure, and then injected with 320 mg/kg pilocarpine and scored for generalized clonus with loss of righting reflex. $NRIF^{-/-}$ mice were compared with control mice of the same strain (Sv129). Because mice are more resistant to neuronal loss after seizures than rats, status epilepticus was allowed to proceed for 2 h before treat-

ment with diazepam. Control animals received all the same treatments except they were injected with saline instead of pilocarpine. During recovery, the animals were treated with Hartman's solution (130 mM NaCl, 4 mM KCl, 3 mM CaCl, 28 mM lactate; 1 ml/100 g) injected subcutaneously twice daily until they were capable of eating and drinking *ad libitum*.

In some cases, rats were cannulated 1 week before the induction of seizures. Rats were anesthetized with ketamine/xylazine, placed in a stereotaxic, and cannulas were implanted bilaterally just dorsal to the hippocampus (anteroposterior, −3.1 mm from bregma; lateral, ±2 mm from midline; dorsoventral, −3 mm from skull). After seizures, anti-pro-NGF (0.5 μl) was infused on one side of the brain and control IgG on the other side twice on the day of seizure, and once daily thereafter until the rats were perfused 3 d after the seizures.

All animal studies were conducted using the National Institutes of Health guidelines for the ethical treatment of animals with approval of the Rutgers Animal Care and Facilities Committee.

Analysis of CSF. At 1, 3, and 7 d after pilocarpine-induced seizure, animals were anesthetized with ketamine/xylazine. CSF (70–100 μl per animal) was collected from the cerebello-medullar cisterna using a 25 gauge butterfly syringe into tubes containing protease inhibitors, snap frozen, and stored at −80°C until analysis. Only CSF samples that did not contain blood contamination were used for Western blot analysis and probed with anti-pro-NGF. To confirm the identity of pro-NGF, 3 μl of MMP7 was added to a 30 μl sample of CSF, incubated at 37°C for 5 min, and then probed in a Western blot.

Immunocytochemistry. Animals were anesthetized with ketamine/xylazine and perfused transcardially with saline followed by 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde for 2 h, and cryoprotected in 30% sucrose overnight. Sections (12 μm) were cut on a cryostat (Leica) and mounted onto charged slides. Sections were blocked in PBS/10% goat serum and permeabilized with PBS/0.3% Triton X-100, and then exposed to primary antibodies overnight at 4°C in PBS/0.3% Triton. Slides were then washed three times in PBS, exposed for 1 h at room temperature to secondary antibodies coupled to different fluorophores, and washed again in PBS in the presence of 4′,6′-diamidino-2-phenylindole (DAPI) (1:500; Sigma-Aldrich) or Draq5 (Alexis Biochemicals) to identify apoptotic neurons. Sections were coverslipped with anti-fading medium (ProLong Gold; Invitrogen) and analyzed by fluorescence microscopy (Nikon). Cultured cells were fixed with 4% paraformaldehyde, blocked for 1 h with PBS/10% normal goat serum, permeabilized with PBS/0.3% Triton X-100 and exposed to primary antibodies overnight at 4°C, washed with PBS, and exposed to secondary antibodies coupled to different fluorophores for 1 h at room temperature. Primary antisera were as follows: anti-pro-NGF and anti-pro-BDNF (Beattie et al., 2002; Volosin et al., 2006), anti-GFAP (Roche), anti-cleaved caspase-3 (1:500; Cell Signaling Technology), anti-p75 (9651; 1:500 dilution) (Huber and Chao, 1995) or 192 IgG (Millipore Bioscience Research Reagents; 1:1000 dilution), and anti-NRIF (Gentry et al., 2004b). No immunostaining was seen in controls with omission of the primary antibodies. To identify dying neurons, cells were also labeled with DAPI and examined by fluorescence microscopy (Stefanis et al.,

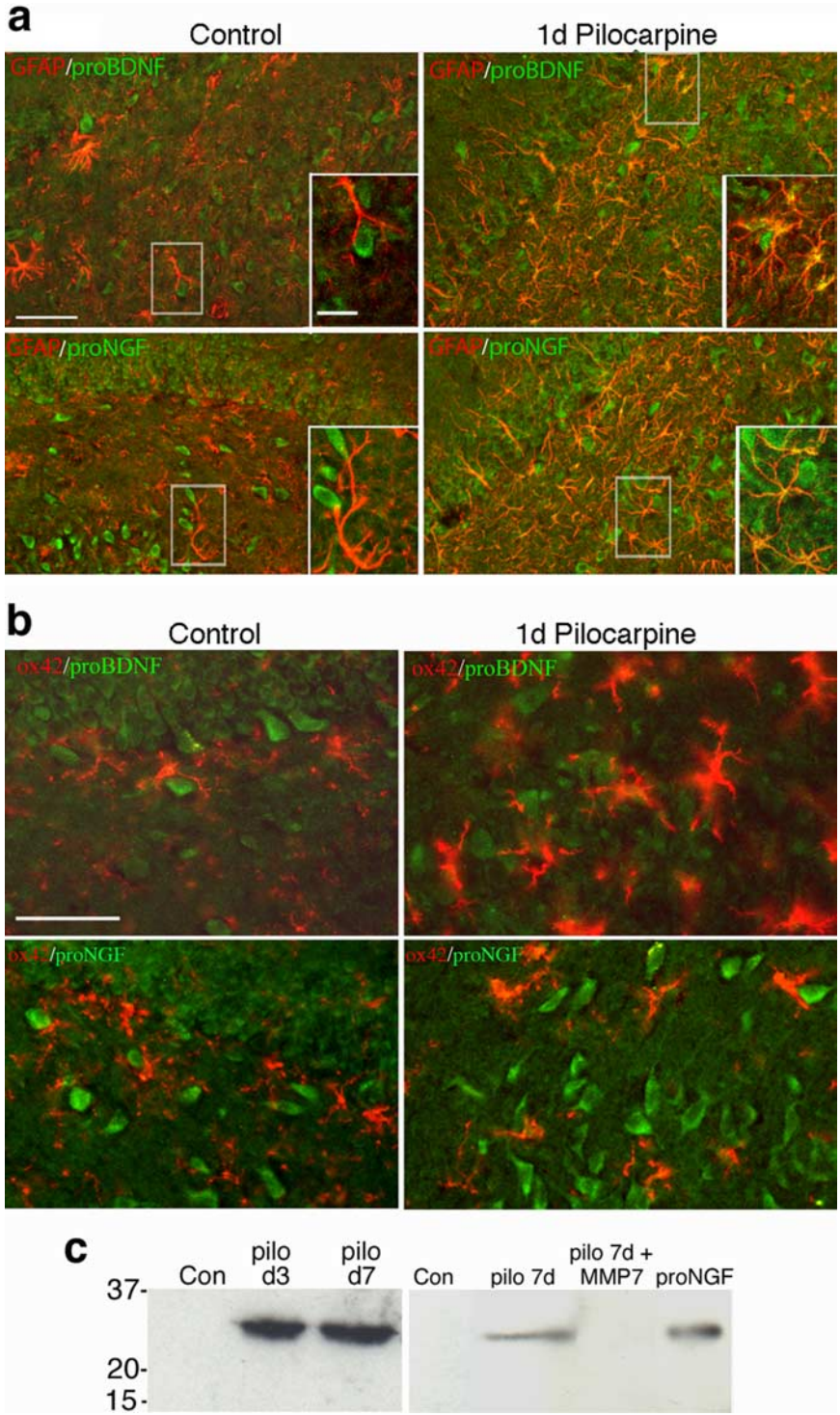

Figure 1. Induction of pro-BDNF and pro-NGF in the hippocampus after seizure. ***a***, Double-label immunostaining for pro-BDNF (top panels) or pro-NGF (bottom panels) and GFAP of rats 1 d after vehicle or pilocarpine treatment. Double labeling for GFAP (red) and proneurotrophins (green) in the dentate gyrus was dramatically increased after seizures. Proneurotrophins were also expressed in hippocampal neurons both before and after seizures. Scale bar, 50 μm; insets, 10 μm. ***b***, Pro-NGF and pro-BDNF were not detected in microglia. Scale bar, 25 μm. ***c***, Pro-NGF was detected in CSF 3 and 7 d after seizures. A total of 30 μl of CSF collected from individual rats treated with either saline or pilocarpine was analyzed by Western blot for pro-NGF. An aliquot of the 7 d CSF was incubated with MMP7, which eliminated the pro-NGF band. Purified pro-NGF was included as reference.

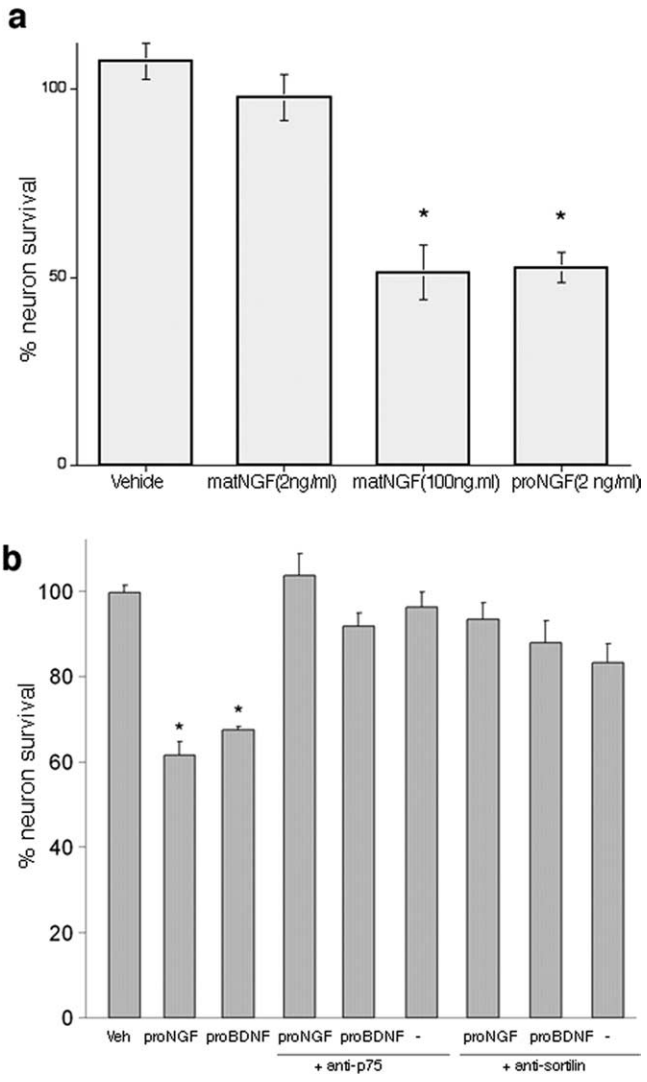

Figure 2. Pro-NGF and pro-BDNF elicit loss of hippocampal neurons in culture. E18 hippocampal neurons were grown for 5 d and treated overnight. ***a***, Pro-NGF elicited the same amount of neuronal loss as mature NGF at a 50-fold lower dose. ***b***, Neuronal death induced by pro-NGF (1 ng/ml) and pro-BDNF (1 ng/ml) was blocked in the presence of antibodies to p75 (9651) or sortilin (Neurotensin receptor 3), each diluted 1:1000 in the medium. The antibodies alone had no effect. Data shown are mean percentage of control ± SEM in triplicate cultures from five independent experiments ($n = 15$). The asterisks indicate values significantly different from vehicle, $p < 0.001$.

1999). Apoptotic nuclei were identified by chromatin condensation and clumping, or by the Nucview caspase-3 substrate (Biotium). Cells and tissue sections were analyzed by epifluorescence (Nikon) or confocal (Carl Zeiss) microscopy. Images were captured digitally and assembled in Adobe Photoshop.

Fluoro-Jade B labeling. The number of dying neurons in wild-type and *NRIF*$^{-/-}$ mice after pilocarpine-induced seizures was assessed by labeling with Fluoro-Jade B according to the published protocol (Schmued and Hopkins, 2000). Labeled neurons were counted in five fields from each of three different sections in the hippocampus from three different animals.

Results

Pro-NGF and pro-BDNF are increased by seizures in the hippocampus *in vivo*

We demonstrated previously that pilocarpine-induced seizures elicit p75^{NTR}-mediated apoptosis of hippocampal neurons (Troy et al., 2002). Recent studies have shown that the preferred ligands for p75^{NTR} are proneurotrophins (Lee et al., 2001; Teng et al., 2005), which have a higher affinity for p75^{NTR} than mature (cleaved) neurotrophins because of the concomitant interaction with sortilin as a coreceptor (Nykjaer et al., 2004). To determine whether proneurotrophins were present in the hippocampus after seizures, rat brains were examined for induction of pro-NGF and pro-BDNF using antibodies that specifically label the prodomains (Beattie et al., 2002; Volosin et al., 2006). Rats were perfused 1, 3, and 7 d after pilocarpine-induced seizures and compared with vehicle-injected control animals. Labeling for both pro-NGF and pro-BDNF was seen in neurons in the control rats and was more widespread at 1 and 3 d after seizures, consistent with previous studies demonstrating seizure-induced BDNF and NGF mRNAs in hippocampal neurons (Gall and Isackson, 1989; Ballarín et al., 1991; Isackson et al., 1991). However, labeling for the proneurotrophins in astrocytes was minimal in the controls and dramatically induced by 1 d after seizure (Fig. 1*a*). Interestingly, pro-NGF and pro-BDNF were not detected in microglia after seizure (Fig. 1*b*). To determine whether the proneurotrophins were secreted, we analyzed CSF from control and pilocarpine-treated rats. In CSF from rats 3 and 7 d after seizures, a band of ~30 kDa was detected, which was eliminated after incubation of the CSF sample with MMP7, an enzyme known to cleave pro-NGF (Lee et al., 2001), suggesting that this proneurotrophin was secreted in the brain after seizures (Fig. 1*c*).

Pro-NGF induces apoptosis of hippocampal neurons *in vitro* and *in vivo*

In our previous studies, we have demonstrated that high concentrations (100 ng/ml) of mature neurotrophins induced p75^{NTR}-mediated apoptosis of hippocampal neurons that lacked the relevant Trk receptor (Friedman, 2000). Because proneurotrophins bind p75^{NTR} with higher affinity than mature neurotrophins, we investigated whether pro-NGF would induce cell death more effectively than the mature neurotrophin. Hippocampal neurons were maintained in culture for 5 d, and then treated overnight with different doses of mature or pro-NGF and compared with vehicle for effects on survival (Fig. 2*a*). Pro-NGF elicited death of hippocampal neurons at 50- to 100-fold lower doses than mature NGF. This effect was blocked by antibodies to p75^{NTR} (9651) (Huber and Chao, 1995; Friedman, 2000) or sortilin (Volosin et al. 2006), confirming that pro-NGF effectively induced apoptosis via a p75^{NTR}/sortilin complex in CNS neurons. Similarly, pro-BDNF induced apoptosis of hippocampal neurons that was blocked by anti-p75 and anti-sortilin (Fig. 2*b*).

To assess whether the pro-NGF induced by seizures in the hippocampus *in vivo* was necessary for neuronal apoptosis, an antibody to the prodomain of pro-NGF was infused into the hippocampus on one side of the brain, while control IgG was infused into the other side for 3 d after pilocarpine treatment. The rats were perfused and analyzed by cleaved caspase-3 (Fig. 3*a*,*b*) or Fluoro-Jade B labeling (Fig. 3*c*,*d*) for dying neurons combined with immunostaining for p75^{NTR} (Fig. 3*e*–*h*). Neuronal loss in the hilus of the dentate gyrus was greatly decreased on the side receiving anti-pro-NGF compared with the contralateral side of the same brain that received control IgG (Fig. 3), demonstrating that endogenous pro-NGF is responsible for most of the seizure-induced neuronal loss. It is interesting to note that anti-pro-NGF also blocked the upregulation of p75^{NTR} that occurs after seizure (Fig. 3*e*,*f*).

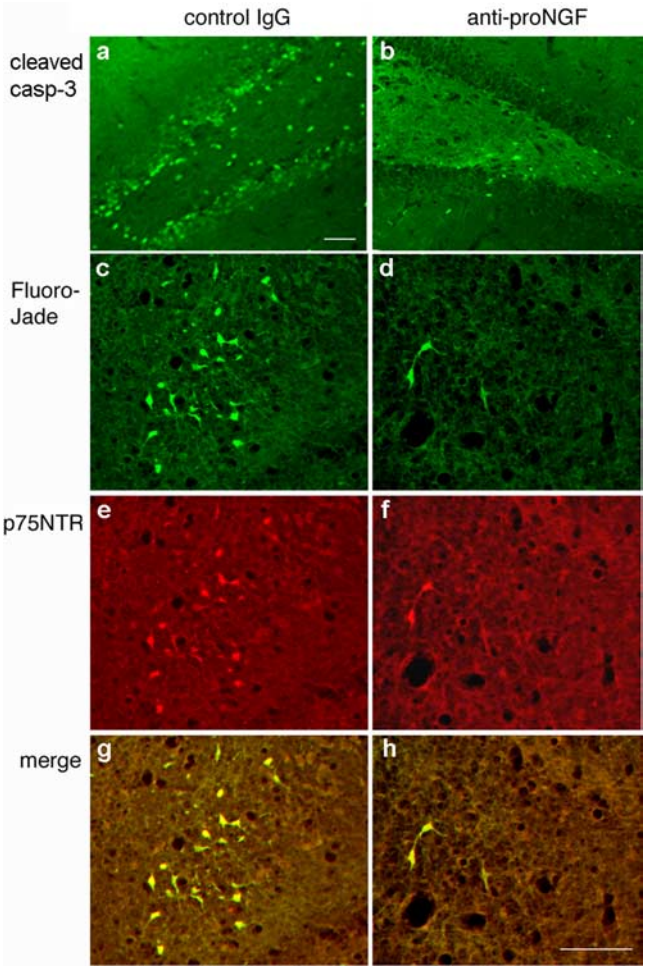

Figure 3. Anti-pro-NGF prevents neuronal loss after seizures *in vivo*. Rats were cannulated bilaterally in the dorsal hippocampus 1 week before seizure. After seizures, anti-pro-NGF (1.5 μg/0.5 μl) was injected on one side and control rabbit IgG on the other side. ***a–h***, Cleaved caspase-3 (***a***, ***b***) and Fluoro-Jade B labeling (***c***, ***d***) combined with immunostaining for p75^{NTR} (***e–h***) demonstrated a decrease in the number of dying neurons with the anti-pro-NGF relative to the control IgG in the same brains. Scale bars: (in ***a***) ***a***, ***b***, 100 μm; (in ***h***) ***c–h***, 50 μm.

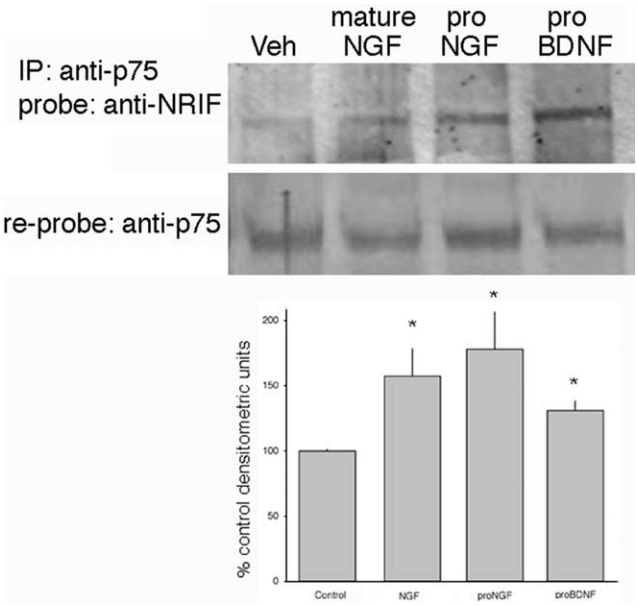

Figure 4. Ligand-induced association of p75^{NTR} and NRIF in hippocampal neurons. Cultured hippocampal neurons were treated for 15 min with vehicle, mature NGF (100 ng/ml), pro-NGF (1 ng/ml), or pro-BDNF (1 ng/ml). Lysates were immunoprecipitated with anti-p75^{NTR} (192 IgG) and probed for NRIF. Blots were stripped and reprobed for p75^{NTR}. The graph shows a densitometric analysis of six different experiments. Error bars indicate SEM. The asterisks indicate values significantly different from control, $p < 0.05$.

NGF and pro-NGF induce NRIF interaction with p75^{NTR} and subsequent NRIF translocation to the nucleus

The p75^{NTR} initiates signaling by recruiting proteins to interact with the intracellular domain of the receptor. To determine whether NRIF could potentially mediate the cell death induced via p75^{NTR} in hippocampal neurons, we investigated whether this protein was recruited to the receptor by ligand treatment. Hippocampal neurons were cultured and treated with vehicle, mature NGF, pro-NGF, or pro-BDNF for 15 min. Coimmunoprecipitation analysis of these neurons demonstrated an increased association of NRIF with p75^{NTR} after treatment with mature NGF (100 ng/ml), pro-NGF (1 ng/ml), or pro-BDNF (1 ng/ml) (Fig. 4). Lower doses of mature NGF (1 ng/ml), which do not elicit apoptosis, did not increase the association of NRIF with p75^{NTR} (data not shown).

In addition to associating with p75^{NTR}, NRIF has been shown to form a complex with the adapter protein TRAF6 (Gentry et al., 2004b), which mediates the ubiquitination and translocation of NRIF to the nucleus in cell lines and sympathetic neurons (Geetha et al., 2005). In contrast, hippocampal neurons treated with NGF or pro-NGF showed no association with TRAF6 (data not shown), consistent with our previous observation that TRAF6 is not expressed in hippocampal neurons (Srinivasan et al., 2004). Because TRAF6 also mediates the activation of NF-κB by p75^{NTR}, these data are consistent with the inability of NGF to activate NF-κB in hippocampal neurons (Fig. 5*A*) in contrast to Schwann cells (Carter et al., 1996). Because TRAF6 also mediates NRIF ubiquitination, which was required for nuclear translocation in sympathetic neurons (Geetha et al., 2005), we investigated whether a different member of the TRAF family could interact with p75^{NTR}. Because TRAF2 is known to mediate TNFα-induced activation of NF-κB (Natoli et al., 1997), which does occur in hippocampal neurons (Fig. 5*A*), we demonstrated that TRAF2 could interact with NRIF on treatment with pro-NGF (Fig. 5*B*). Moreover, treatment of hippocampal neurons with pro-NGF induced ubiquitination of NRIF despite the absence of TRAF6 (Fig. 5*C*), suggesting that a different ubiquitin ligase, possibly TRAF2, functions in these neurons.

The NRIF protein has been suggested to function as a transcription factor based on structural characteristics and its ability to translocate to the nucleus (Gentry et al., 2004b). We examined the intracellular localization of NRIF in hippocampal neurons at different time points after treatment with pro-NGF by immunostaining and confocal microscopy. Cultured neurons were treated with pro-NGF for 15 min, 30 min, 1 h, 2 h, or 4 h. Nuclear localization of NRIF was detected in p75^{NTR}-positive cells after 1 h treatment (Fig. 6*A*) and remained in the nucleus for at least 2 h. By 4 h after treatment, NRIF localization was again entirely cytoplasmic (data not shown).

Cleavage of p75^{NTR} is required for NRIF nuclear translocation and apoptotic signaling

Several studies have suggested that p75^{NTR} can undergo regulated intramembrane proteolysis (Jung et al., 2003; Zampieri et al., 2005; Urra et al., 2007), and this cleavage is necessary for NRIF nuclear translocation in sympathetic neurons (Kenchappa et al.,

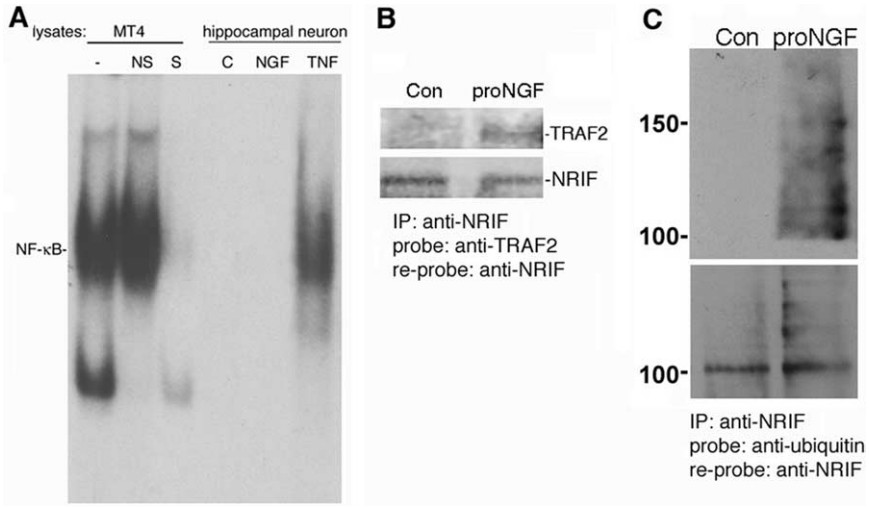

Figure 5. p75^{NTR} signaling is different in hippocampal neurons compared with other cell types. ***A***, NGF does not activate NF-κB in hippocampal neurons. Electrophoretic mobility shift assay analysis of NF-κB binding in nuclear extracts from cultured hippocampal neurons is shown. Nuclear extracts were prepared from cultured neurons that were treated for 1 h with vehicle, NGF (100 ng/ml), or TNF (10 ng/ml). Nuclear extracts from MT4 cells were used as positive controls and to establish specificity. Nuclear extracts were incubated with a ^{32}P-labeled oligonucleotide probe consisting of a consensus NF-κB sequence. NF-κB indicates the band that is eliminated by fivefold excess specific (S) competing oligonucleotide but not by a nonspecific (NS) oligonucleotide. ***B***, Coimmunoprecipitation showing ligand-induced association of TRAF2 with NRIF. ***C***, NRIF is ubiquitinated after pro-NGF treatment. Hippocampal neurons treated with pro-NGF for 30 min were lysed, immunoprecipitated for NRIF, and probed on a Western blot for ubiquitin, stripped, and reprobed for NRIF.

2006). To assess whether receptor cleavage was necessary for the translocation of NRIF to the nucleus and apoptotic signaling in CNS hippocampal neurons, cells were treated with vehicle, NGF (100 ng/ml), or pro-NGF (1 ng/ml) in the absence or presence of the α-secretase inhibitor TNFα protease inhibitor (TAPI) or the γ-secretase inhibitor DAPT, and the neurons were evaluated for effects on NRIF nuclear translocation and neuronal survival. The presence of TAPI or DAPT attenuated the induction of apoptosis by NGF (100 ng/ml) or pro-NGF (1 ng/ml) (Fig. 6*B*), and also blocked the translocation of NRIF to the nucleus (Fig. 6*A*). The inhibition of NRIF nuclear localization by TAPI is quantified in Figure 6*C* and was similarly inhibited by DAPT (data not shown). Moreover, nuclear NRIF was correlated with caspase activity (Fig. 6*D*), suggesting that the cleavage of p75^{NTR} is a critical step in apoptotic signaling in hippocampal neurons.

Ligand-induced association of NRIF and NRAGE

Several p75^{NTR} binding proteins in addition to NRIF have been implicated in apoptotic signaling, in particular NRAGE (Salehi et al., 2002). To assess whether these p75^{NTR} binding proteins may function together to activate apoptotic signaling, we investigated whether NRIF and NRAGE interact in hippocampal neurons, either constitutively or on ligand stimulation. Cultured hippocampal neurons were treated with vehicle, mature NGF (100 ng/ml), or pro-NGF (1 ng/ml). Coimmunoprecipation analysis demonstrated a ligand-induced interaction between NRIF and NRAGE (Fig. 7), suggesting that several of the identified p75^{NTR}-binding proteins may function together to initiate apoptotic signaling.

Requirement of NRIF for p75^{NTR}-mediated apoptosis of hippocampal neurons *in vitro* and *in vivo*

To assess whether NRIF was required for p75^{NTR}-mediated apoptosis of hippocampal neurons, cultures were prepared from wild-type, *NRIF*$^{+/-}$, or *NRIF*$^{-/-}$ mice and exposed overnight to NGF (100 ng/ml) or pro-NGF (1 ng/ml). In the wild-type mouse neurons, treatment with either ligand induced loss of 70–80% of the population; however, the hippocampal neurons from *NRIF*$^{-/-}$ mice were completely resistant, and the heterozygotes were partially resistant, to death induced by either NGF or pro-NGF (Fig. 8), demonstrating that NRIF was required to mediate apoptosis induced via p75^{NTR}.

To examine the potential role of NRIF in mediating p75^{NTR}-activated neuronal death *in vivo*, we used pilocarpine-induced seizures, which elicits p75^{NTR}-mediated apoptosis of hippocampal neurons (Troy et al., 2002). In the normal adult rat hippocampus, p75^{NTR} expression was not observed, consistent with previous studies (Roux et al., 1999; Troy et al., 2002), and NRIF expression was localized to the cytosol of hippocampal neurons (Fig. 9*Aa*). After pilocarpine treatment, there was a significant threefold increase in nuclear NRIF in p75^{NTR}-positive neurons (Fig. 9*Ab*).

To assess the need for NRIF in signaling apoptosis in this paradigm, wild-type and *NRIF*$^{-/-}$ mice were subjected to pilocarpine-induced seizures. Double-label immunofluorescence was used to investigate the colocalization of cleaved caspase-3 and p75^{NTR} in the hippocampus. Wild-type mice injected with saline had no p75^{NTR} or cleaved caspase-3 labeled neurons, as we have shown previously for adult rats and mice (Troy et al., 2002), although blood vessels were strongly p75^{NTR}-positive (Fig. 9*Ba*). However, by 1 d after seizure, there were numerous p75^{NTR}-positive neurons that showed abundant cleaved caspase-3 labeling in wild-type mice, particularly in the dentate gyrus (Fig. 9*Bb*), suggesting that the p75^{NTR}-positive neurons were apoptotic. In contrast to wild-type mice, basal levels of p75^{NTR} were increased in the hippocampus of control *NRIF*$^{-/-}$ mice (Fig. 9*Bc*). The *NRIF*$^{-/-}$ mice also showed increased expression of p75^{NTR} 1 d after seizure; however, these neurons did not have cleaved caspase-3 (Fig. 9*Bd*), suggesting that p75^{NTR}-mediated apoptotic signaling was deficient in the absence of NRIF. Fluoro-Jade B labeling was also used to identify dying neurons in the wild-type and *NRIF*$^{-/-}$ mice and revealed that neuronal death was decreased in the *NRIF*$^{-/-}$ mice by 75% relative to wild type after seizures, similar to the 80% decrease in neuronal loss we previously observed in *p75*$^{NTR-/-}$ mice (Troy et al., 2002).

Discussion

The p75^{NTR} can mediate many different cellular functions; however, the mechanisms by which this receptor signals remain enigmatic. An important role for p75^{NTR} in mediating cell death in the developing nervous system has been demonstrated in the retina (Frade et al., 1996), in sympathetic neurons (Bamji et al., 1998), and in basal forebrain cholinergic neurons (Naumann et al., 2002). Injury to the CNS can also elicit p75^{NTR}-mediated apoptosis of oligodendrocytes (Beattie et al., 2002), cortical neurons (Harrington et al., 2004), hippocampal neurons (Friedman, 2000; Troy et al., 2002), and basal forebrain neurons (Volosin et al., 2006). In this study, we demonstrate that NRIF is a critical signaling component of the p75^{NTR} apoptotic signaling pathway for hippocampal neurons both in primary culture and *in vivo* to mediate neuronal death after seizures.

Recent studies have demonstrated that proneurotrophins bind with higher affinity to p75^{NTR} than do mature neurotrophins (Lee et al., 2001; Teng et al., 2005), because of the concomitant binding with sortilin as a coreceptor (Nykjaer et al., 2004). Although mature neurotrophins can induce p75^{NTR}-mediated apoptosis of hippocampal neurons (Friedman, 2000), pro-NGF was 50- to 100-fold more potent than NGF in inducing apoptosis *in vitro*.

Proneurotrophins *in vivo*

Because proneurotrophins are the preferred ligands for induction of p75^{NTR}-mediated apoptosis, we investigated whether the presence of proneurotrophins in the hippocampus was altered after seizures, which we (Troy et al., 2002), and others (Roux et al., 1999) have shown elicits expression of p75^{NTR} and subsequent apoptosis. Previous studies had shown that seizures caused increases in BDNF and NGF mRNAs in hippocampal neurons (Gall and Isackson, 1989; Ballarín et al., 1991; Isackson et al., 1991). A variety of other insults also induce NGF mRNA in astrocytes (DeKosky et al., 1994; Oderfeld-Nowak and Bacia, 1994; Goss et al., 1998); however, none of these studies determined which form of the protein was produced. We investigated whether there was an increase in pro-NGF and pro-BDNF protein in the hippocampus after seizure. Antibodies that specifically recognize the prodomains of these factors (Beattie et al., 2002; Volosin et al., 2006) showed that pro-BDNF and pro-NGF were more widespread in hippocampal neurons, and were dramatically induced in hippocampal astrocytes after seizures. Moreover, the presence of pro-NGF in the CSF demonstrated that pro-NGF was released after the seizure, similar to what was shown after a corticospinal lesion (Harrington et al., 2004). Thus, there is an abundance of proneurotrophins present in the hippocampus after seizure. The infusion of anti-pro-NGF dramatically decreased the number of dying neurons compared with infusion of control rabbit IgG, demonstrating that the release of endogenous pro-NGF plays a key role in neuronal loss after seizures. Anti-pro-NGF infusion also prevented the upregulation of p75^{NTR} in the hippocampus after seizure, suggesting that the ligand regulates induction of the receptor before eliciting apoptosis.

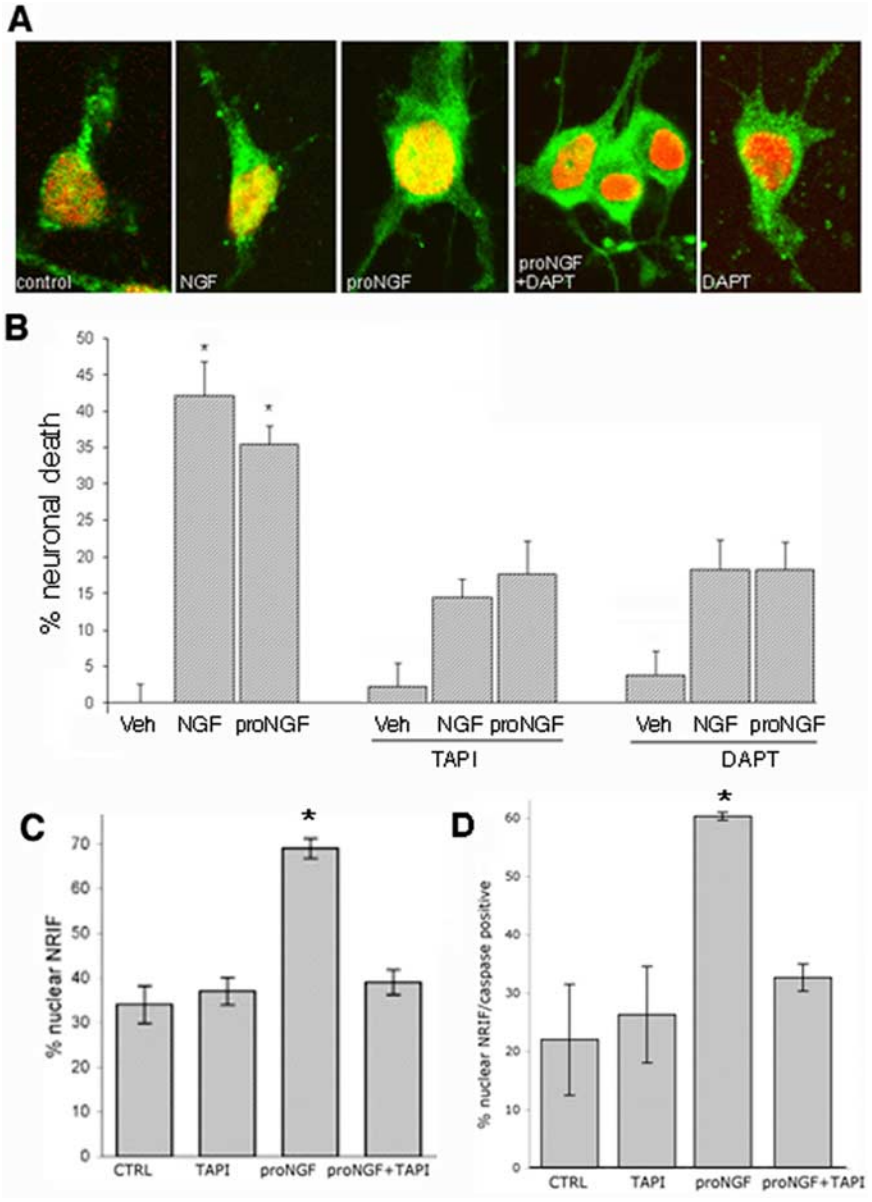

Figure 6. p75^{NTR} cleavage is necessary for apoptotic signaling and NRIF nuclear translocation in hippocampal neurons. ***A***, Confocal microscopy analysis of NRIF localization (green) in hippocampal neurons with Draq5 (red) as the nuclear marker. NGF and pro-NGF induced NRIF nuclear localization. Inhibition of p75^{NTR} cleavage with TAPI or DAPT prevented pro-NGF-induced nuclear localization of NRIF (shown for DAPT). ***B***, Hippocampal neurons were grown for 5 d and treated overnight with NGF (100 ng/ml) or pro-NGF (1 ng/ml) in the absence or presence of the α-secretase inhibitor TAPI (10 μM) or the γ-secretase inhibitor DAPT (2 μM). Both inhibitors prevented ligand-induced death. Experiments were conducted in triplicates in five independent experiments ($n = 15$). Data are expressed as percentage apoptotic neurons relative to vehicle-treated controls. Statistical significance was determined by one-way ANOVA and Tukey's *post hoc* analysis. The asterisks indicate values significantly different from vehicle, $p < 0.001$. ***C***, Hippocampal neurons were cultured for 5 d and treated with pro-NGF in the presence or absence of TAPI, fixed, and immunostained for NRIF and p75^{NTR}. Data are expressed as the percentage of p75^{NTR}-positive neurons with nuclear NRIF. Statistical significance was determined by one-way ANOVA and Tukey's *post hoc* analysis. The asterisk indicates a significant difference from vehicle, $p < 0.005$. ***D***, Nuclear NRIF localization was correlated with cell death. Cultured hippocampal neurons were treated as indicated, fixed, and double labeled with anti-NRIF and the Nucview caspase-3 substrate. The asterisk indicates a value significantly different from vehicle, $p < 0.05$. Error bars indicate SEM.

Role of NRIF in p75^{NTR}-mediated apoptosis

Activation of p75^{NTR} leads to phosphorylation of JNK (Friedman, 2000; Harrington et al., 2002) and the phosphorylation of Bad (Bhakar et al., 2003), with the consequent activation of the intrinsic caspase pathway using caspases-9, -6, and -3 (Wang et al., 2001; Troy et al., 2002). However, signaling events proximal to the receptor remain poorly defined, especially in a physiological context. Recently, a role for the p75 ^{NTR}-binding protein NRIF was demonstrated for sympathetic neurons, requiring cleavage of the p75^{NTR}, ubiquitination and translocation of NRIF to the nucleus (Kenchappa et al., 2006). We have investigated a

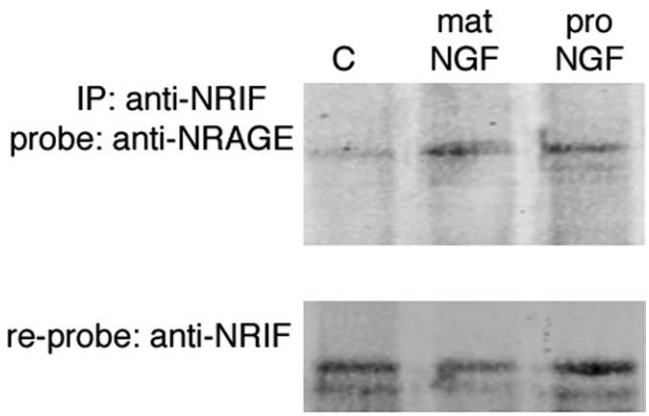

Figure 7. Interaction of NRIF and NRAGE. Hippocampal neurons were treated for 15 min with vehicle, mature NGF (100 ng/ml), or pro-NGF (1 ng/ml). Lysates were immunoprecipitated with anti-NRIF and probed by Western blot for NRAGE. Blots were stripped and reprobed for NRIF.

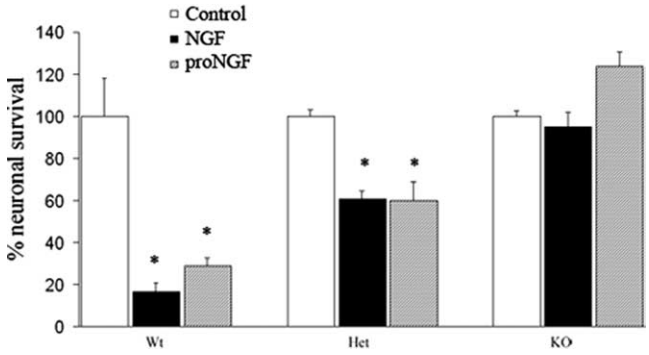

Figure 8. *NRIF* $^{-/-}$ neurons are resistant to p75^{NTR}-mediated cell death. Cultured hippocampal neurons were prepared from wild-type, *NRIF* $^{+/-}$, or *NRIF* $^{-/-}$ mice and treated overnight with vehicle, mature NGF (100 ng/ml), or pro-NGF (1 ng/ml). The asterisks indicate values significantly different from untreated controls, $p < 0.05$. Error bars indicate SEM.

potential role for NRIF in mediating proneurotrophin-induced apoptosis of hippocampal neurons. We have demonstrated that treatment of hippocampal neurons with ligand (either 100 ng/ml NGF or 1 ng/ml pro-NGF) induced an increased association of NRIF with p75^{NTR}, and subsequent ubiquitination and translocation of NRIF to the nucleus. Moreover, hippocampal neurons cultured from *NRIF* $^{-/-}$ mice were resistant to pro-NGF-induced cell loss, demonstrating the requirement for this protein to mediate apoptotic signaling via p75^{NTR}.

Cell-specific p75^{NTR} signaling

The p75^{NTR} has been implicated in many functions in different cell types; however, cell-specific differences in p75^{NTR} signaling have not been characterized. Previous studies have demonstrated that p75^{NTR}-dependent apoptosis in sympathetic neurons requires cleavage of the receptor, binding of NRIF to TRAF6, which mediates its ubiquitination, and nuclear translocation of NRIF (Gentry et al., 2004b; Geetha et al., 2005; Kenchappa et al., 2006). In the present study, we demonstrate that p75^{NTR}-mediated apoptotic signaling in hippocampal neurons also requires cleavage of the receptor and nuclear translocation of NRIF; however, no association with TRAF6 was detected. This result is consistent with our previous demonstration that hippocampal neurons lack TRAF6 and do not activate NF-κB in response to IL-1β (Srinivasan et al., 2004). Consistent with the lack of TRAF6, NGF failed to activate NF-κB in hippocampal neurons, indicating important cell specific differences in p75^{NTR} signaling, because activation of this receptor does lead to activation of NF-κB in Schwann cells (Carter et al., 1996). However, despite the lack of TRAF6, we observed that NRIF was ubiquitinated in hippocampal neurons before nuclear translocation, as in sympathetic neurons. Consistent with the ability of TNFα to activate NF-κB in hippocampal neurons, TRAF2 interacted with NRIF, and may functionally replace TRAF6 in ubiquitinating NRIF in these neurons.

Cleavage of p75^{NTR} was required for NRIF nuclear translocation and cell death to occur in the hippocampal neurons as shown previously for sympathetic neurons. Interestingly, however, the time course for p75^{NTR} cleavage, and the ubiquitination and nuclear translocation of NRIF was different in the hippocampal neurons than sympathetic neurons. Sympathetic neurons exhibited these events over a more protracted time course (Kenchappa et al., 2006), with NRIF detected in the nucleus 30 h after ligand treatment. In contrast, we detected NRIF translocation to the nucleus in the hippocampal neurons within 1 h. Because the NRIF translocation is dependent on p75^{NTR} cleavage, one possibility is that the secretases required for receptor cleavage may be constitutively expressed in hippocampal but not sympathetic neurons.

Interaction of NRIF and NRAGE

Several p75^{NTR}-binding proteins have been identified that have been implicated in cell death, in particular NRIF and NRAGE, but how these proteins mediate signaling is not understood. Both NRIF and NRAGE have been implicated in activation of JNK (Salehi et al., 2002; Linggi et al., 2005); therefore, we assessed whether these p75^{NTR}-binding proteins might interact in a complex to activate a common pathway. Coimmunoprecipitation analysis demonstrated that ligand treatment increased the association between NRIF and NRAGE, suggesting that several p75^{NTR}-binding proteins may interact in a complex to stimulate apoptotic signaling in these hippocampal neurons.

Role of NRIF *in vivo*

In vivo, seizures induced by pilocarpine (Roux et al., 1999; Troy et al., 2002) or kainic acid (Volosin et al., 2006) induced p75^{NTR}-mediated neuronal apoptosis. In the present study, we demonstrated that in hippocampal neurons of control rats NRIF was localized to the cytoplasm; however, after seizures, NRIF could be detected in nuclei of p75^{NTR}-positive neurons. Moreover, in *NRIF* $^{-/-}$ mice, neuronal loss in the hippocampus was attenuated compared with wild-type mice, similar to what we have previously shown for *p75* $^{-/-}$ mice (Troy et al., 2002). Specifically, neurons positive for p75^{NTR} were not apoptotic in the *NRIF* $^{-/-}$ mice, confirming the critical role for NRIF in mediating p75^{NTR}-dependent cell death. Interestingly, *NRIF* $^{-/-}$ mice that were not treated with pilocarpine had p75^{NTR}-positive neurons in the hippocampus, which were not seen in wild-type mice, suggesting that these neurons may have been spared from developmental cell death because of the lack of NRIF.

In conclusion, we demonstrate in these studies that proneurotrophins are abundantly present in the hippocampus *in vivo*, are strongly upregulated in astrocytes after seizures, and mediate neuronal loss under these conditions. Pro-NGF and pro-BDNF potently induce apoptosis of hippocampal neurons *in vitro* by a mechanism that requires p75^{NTR} cleavage, ubiquitination and nuclear translocation of NRIF, which was required for p75^{NTR}-mediated apoptosis of hippocampal neurons both *in vitro* and *in*

vivo after seizures. We further demonstrate that the signaling mechanisms activated by p75^{NTR} in hippocampal neurons are distinct from other cell types.

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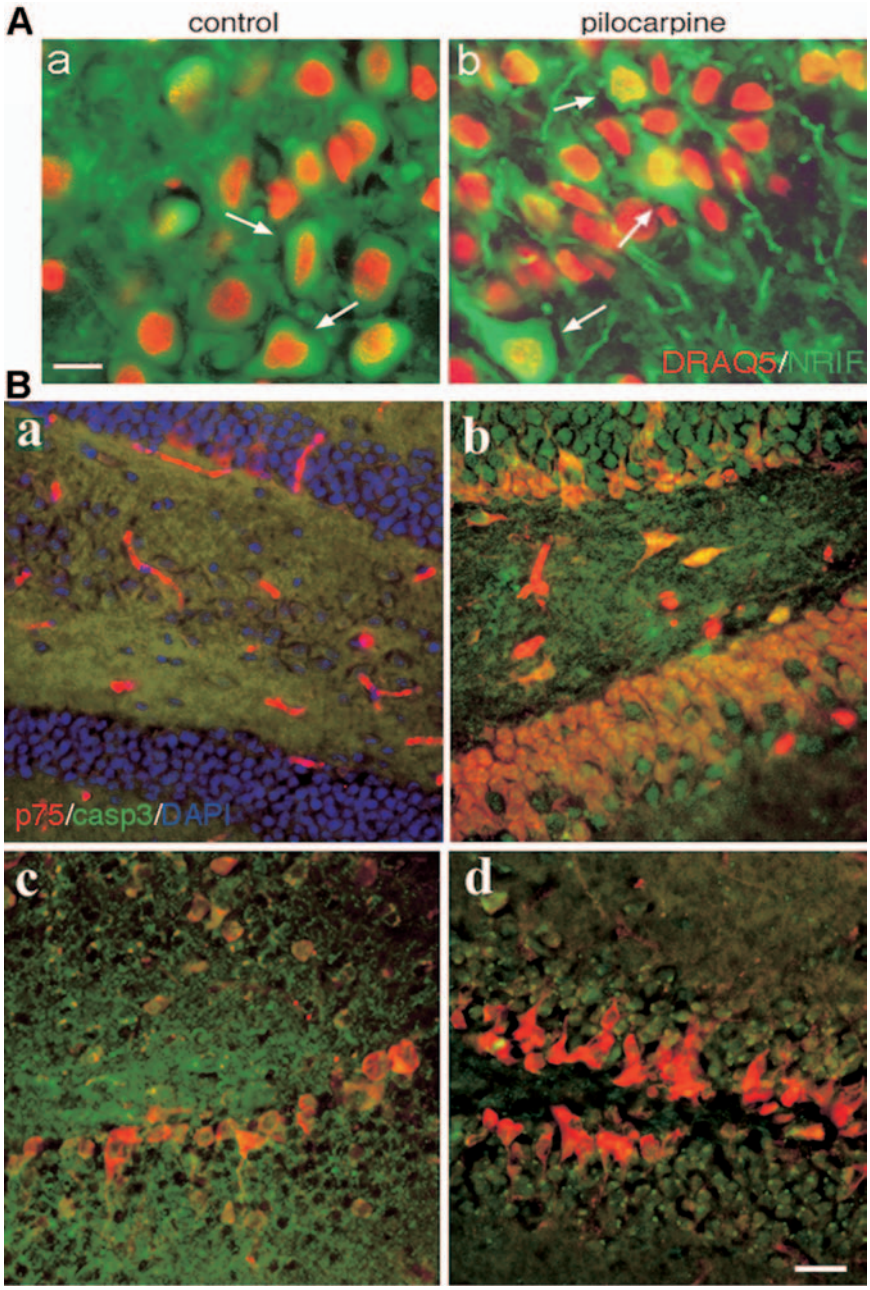

Figure 9. NRIF is translocated to the nucleus and mediates neuronal loss after seizures *in vivo*. ***A***, Immunostaining of hippocampal neurons for NRIF (green) after vehicle (***a***) or pilocarpine injection (***b***). Nuclei are labeled with Draq5 (red). Note the cytoplasmic localization of NRIF in the vehicle-treated rats, and the nuclear localization (yellow) after pilocarpine. Scale bar: (in ***a***) ***a***, ***b***, 10 μm. ***B***, p75^{NTR}-positive neurons in *NRIF*$^{-/-}$ mice are resistant to cell death *in vivo*. Wild-type (***a***, ***b***) or *NRIF*$^{-/-}$ (***c***, ***d***) mice after vehicle (***a***, ***c***) or pilocarpine-induced seizures (***b***, ***d***) were immunostained for p75^{NTR} and cleaved caspase-3. DAPI labeling in ***a*** shows the location of the dentate gyrus. Seizures induced extensive expression of p75^{NTR} in wild-type hippocampal neurons that also were labeled for cleaved caspase-3 (***b***; note yellow/orange labeling), indicative of apoptotic cells. In contrast, seizures in the *NRIF*$^{-/-}$ mice induced p75^{NTR} expression without cleaved caspase-3 labeling (***d***). Note the p75^{NTR}-positive neurons in the control *NRIF*$^{-/-}$ hippocampus (***c***) compared with the wild-type control in which only blood vessels are labeled for p75^{NTR} (***a***). Scale bar: (in ***d***) ***a–d***, 100 μm.

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